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### **Multilocus analysis in candidate genes ACE, AGT, and AGTR1 and predisposition to peripheral arterial disease: role of ACE D/-240T**

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# Multilocus analysis in candidate genes *ACE*, *AGT*, and *AGTR1* and predisposition to peripheral arterial disease: Role of *ACE* D/-240T haplotype

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**Objective:** Peripheral arterial disease (PAD) is a common manifestation of systemic atherosclerosis. Apart from traditional cardiovascular risk factors, several novel biologic mediators and genetic predisposing factors appear relevant in determining the atherogenetic process leading to PAD. Genes encoding for renin angiotensin system (RAS) components have been proposed as candidate in atherosclerosis. This study investigated four polymorphisms in angiotensinogen (*AGT*), angiotensin converting enzyme (*ACE*), and angiotensin II receptor type 1 (*AGTR1*), genes of RAS, in both predicting PAD and modulating the severity of the disease.

**Methods:** The *ACE* I/D and -240A>T, *AGT* M235T, and *AGTR1* 1166A>C polymorphisms were analyzed in 281 PAD patients and in 485 controls comparable for age and sex.

**Results:** The *ACE* D and -240T alleles both significantly influenced the predisposition to PAD. The *ACE* D, but not -240 T, allele remained associated with PAD after Bonferroni correction ( $P = .004$ ) and adjustment for cardiovascular risk factors ( $P = .03$ ). The *ACE* D allele influenced PAD predisposition with a dose-dependent effect (odds ratio for *ACE* ID vs II genotype, 1.77;  $P = .006$ ; *ACE* DD vs II genotype, 2.15;  $P = .001$ ). The haplotype reconstruction analysis for the *ACE* gene showed that the D/-240T haplotype significantly and independently influenced the predisposition to PAD ( $P = .02$ ). In 190 PAD patients with no additional atherosclerotic localizations (isolated PAD), a significant association between *ACE* D and -240T alleles and PAD was observed. Only the *ACE* D allele remained associated with isolated PAD after Bonferroni correction ( $P = .02$ ) and after adjustment for cardiovascular risk factors ( $P = .02$ ). The haplotype reconstruction analysis for the *ACE* gene showed that the D/-240T, but not the D/-240A haplotype significantly influenced the predisposition to PAD ( $P = .0003$ ). No influence of the polymorphisms analyzed on the severity of the disease, according to Rutherford categories, was found.

**Conclusions:** The present study contributes data to highlight the role of the *ACE* D/-240T haplotype in predisposing to PAD, also in the absence of other atherosclerotic comorbidities. (J Vasc Surg 2009;50:1399-404.)

Peripheral arterial disease (PAD) is a common manifestation of systemic atherosclerosis and is associated with an increased risk of cardiovascular events related to the coexistence of coronary artery disease (CAD) and cerebrovascular disease (CVD).<sup>1</sup> Apart from traditional cardiovascular risk factors, several novel biologic mediators and genetic predisposing factors appear relevant in determining the atherogenetic process that leads to PAD.<sup>1-3</sup> PAD is indeed a complex disorder. Its pathogenesis is the result of the interaction between multiple genes and multiple environmental factors and is not yet completely defined. However, information is increasing on the relevance of investigating

polymorphisms in candidate genes, such as renin angiotensin system (RAS) genes and the methylenetetrahydrofolate reductase (*MTHFR*) gene. These genes are involved in homocysteine metabolism and their association with the predisposition to PAD has been demonstrated.<sup>2,3</sup>

The involvement of the RAS in atherosclerosis has been demonstrated,<sup>4</sup> and genes encoding for its components, such as angiotensinogen (*AGT*), angiotensin-converting enzyme (*ACE*), and angiotensin II receptor type 1 (*AGTR1*), have been proposed as candidates that influence the predisposition to both CAD and CVD.<sup>5,6</sup> The few published reports on the relationship between RAS genes and PAD have had conflicting results.<sup>2,7,8</sup> A recent hypothesis is that people carrying susceptibility polymorphisms in RAS genes might exhibit higher angiotensin II levels, which may be crucial in the development of the disease.

The *M235T* polymorphism in the *AGT* gene, which consists of a nucleotide substitution in exon 2 that leads to the methionine → threonine substitution at position 235 in the amino acid sequence, is associated with increased concentrations of angiotensinogen in circulating blood.<sup>9</sup> It has been speculated that increased plasma or tissue angiotensinogen level associated with the *AGT* 235T variant may stem from alterations in different steps of the metabolic pathway, which could lead to a small increase in baseline

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levels or in the production of angiotensin II.<sup>9</sup> An association of the *AGT* M235T polymorphism with hypertension<sup>9</sup> and myocardial infarction<sup>10</sup> has been demonstrated.

In the intron 16 of the gene encoding for ACE, a polymorphism consisting of an insertion/deletion (I/D) of a 287-bp fragment has been identified,<sup>11</sup> and the ACE D allele has been reported to be associated with increased serum levels of the circulating enzyme.<sup>11</sup> The ACE DD genotype is associated with higher plasma levels of the enzyme, the II genotype with lower ACE levels, and the ID genotype with intermediate levels. Experimental studies have reported a functional role for the ACE I/D polymorphism in modulating angiotensin II levels<sup>12</sup> as well as an increased messenger RNA (mRNA) expression in white blood cells from individuals carrying the ACE D allele compared with those carrying the I allele.<sup>13</sup>

The role of the ACE I/D polymorphism in affecting atherosclerosis is of interest. We have previously demonstrated that the ACE D allele represents a susceptibility factor not only for CAD<sup>14</sup> but also for other atherosclerotic localizations such as abdominal aortic aneurysm (AAA).<sup>15</sup>

Other functional polymorphisms in the promoter region of the ACE gene (-240A>T) and in the gene encoding for angiotensin II type 1 receptor (*AGTR1* A1166C) have been analyzed in atherosclerotic cardiovascular disease,<sup>16</sup> and the *AGTR1* polymorphism has been demonstrated to influence aortic stiffness<sup>17</sup> and vascular reactivity,<sup>18</sup> but no data are available concerning their role in PAD.

Because PAD is a complex disorder in which environmental and genetic components interact in determining its pathogenesis, we investigated the genetic influence of four polymorphisms in RAS genes—ACE, *AGT*, and *AGTR1*—as predisposing factors to PAD and as markers potentially involved in the modulation of the severity of the disease.

## METHODS

**Study population.** We studied 281 patients with symptoms or signs suggestive for the presence of PAD who were referred to the Unit of Vascular Surgery of the University of Florence to be evaluated for possible surgical intervention, in part analyzed in a previous study.<sup>19</sup> This is a retrospective case-control association study. PAD was diagnosed when patients had typical symptoms of intermittent claudication, such as cramping pain of the calves or buttocks during exercise, and an ankle-brachial index at rest of <0.90, calculated according to the recommendations of the American Heart Association.<sup>20</sup>

All patients were also evaluated for atherosclerotic disease at other locations. In particular, all patients underwent a cardiologic evaluation that included an electrocardiogram and echocardiogram. In patients with symptoms potentially related to ischemic heart disease, additional studies included echocardiogram with drug-induced stress testing, myocardial scintigraphy, and coronary angiography. Carotid artery duplex ultrasound scanning with color-coded echo flow imaging was also done. Patients with associated familial and inflammatory AAAs were excluded from the

study. Rutherford categories was assigned as follow: class 2, moderate claudication; class 3, severe claudication; class 4, rest pain; class 5 to 6, ulcers or gangrene.

The patients were compared with 485 clinically healthy volunteers recruited from the staff of the University of Florence and the hospital, and from partners or friends of patients. The control group was selected to be comparable for age and sex with the patient group. As part of a physical examination, expert physicians performed a detailed interview addressing personal and familial history to identify disease-free controls and to exclude individuals who were suspected of having any form of vascular disease.

The participants were considered to have hypertension if they had been diagnosed as being hypertensive according to the guidelines of European Society of Hypertension/European Society of Cardiology<sup>21</sup> or were taking antihypertensive drugs. Dyslipidemia was defined according to the Third Report of the National Cholesterol Education Program (NCEP),<sup>22</sup> and diabetes in agreement with the American Diabetes Association.<sup>23</sup> A positive family history was defined as the presence of at least one first-degree relative in whom cardiovascular disease had developed age <55 years for men and <65 years for women. All participants gave informed consent. The study complies with the Declaration of Helsinki and was approved by the local ethics committee.

**Detection of RAS polymorphisms.** Genomic DNA extraction was performed from peripheral blood leucocytes by using a QIAmp Blood Kit (QIAGEN, Hilden, Germany).

Previously reported methods<sup>14,24</sup> were used to genotype the following RAS polymorphisms:

- ACE I/D (rs4340; NT\_010783.14:g.20217903\_20217904ins5),
- ACE -240A>T (rs4291; NT\_010783.14:g.20206205T>A),
- *AGTR1* 1166A>C (rs5186; NT\_005612.15:g.54955134A>C), and
- *AGTM*235T (rs699; NT\_004559.13:g.7047947A>G).

**Statistical analysis.** Statistical analysis was performed with SPSS 11.5 software (SPSS Inc, Chicago, Ill). Continuous variables are expressed as median (range). The non-parametric Mann-Whitney test for unpaired data was used for comparison between single groups, as appropriate. The  $\chi^2$  test was used to test for deviation of genotype distribution from Hardy-Weinberg equilibrium, to evaluate the differences in genotype distribution and allele frequency, and to analyze the prevalence of traditional risk factors between patients and controls.

Logistic regression was used to analyze the association between ACE, *AGTR1*, and *AGT* polymorphisms and PAD under a dominant genetic model of inheritance that compares individuals with one or more polymorphic alleles with a baseline group with no polymorphic alleles (eg ACE DD+ID vs II). The number of participants studied was sufficient to detect, with a statistical power of 80% ( $\beta = 0.8$ ) and significance at  $\alpha = 0.05$ , absolute differences in ACED

**Table I.** Demographic and clinical characteristics of the study populations

Variable	Patients (n = 281) Mean (range), No. (%)	Controls (n = 485) Mean (range), No. (%)	P
Age, y	72 (30-93)	71 (24-95)	.4
Males	220 (78)	360 (74)	.2
Hypertension	167 (59.4)	99 (20)	<.0001
Diabetes	47 (16.7)	25 (5.2)	<.0001
Dyslipidemia	135 (48)	96 (19.8)	<.0001
Smoking habit	176 (62.6)	102 (21)	<.0001
Family history <sup>a</sup>	56 (19.9)	29 (5.9)	<.0001
CAD	70 (24.9)	...	...
AAA	34 (12.1)	...	...
CVD	28 (10.0)	...	...
Rutherford category			
2	28 (9.9)	...	...
3	188 (66.9)	...	...
4	48 (17.1)	...	...
5-6	17 (6.0)	...	...

AAA, Abdominal aortic aneurism; CAD, coronary artery disease; CVD, cerebrovascular disease.

<sup>a</sup>Family history of cardiovascular disease.

allele frequencies between patients and controls. A value of  $P < .05$  was considered to indicate statistical significance.

The Bonferroni correction was used for multiple testing (the four candidate polymorphisms were treated as four independent statistical tests) by multiplying the nominal  $P$  value of each test by the number of tests conducted.

Haplotype reconstruction and frequency estimation were performed using the R (<http://www.r-project.org>) package haplo.stats by Expectation-Maximization strategy (EM algorithm).<sup>25</sup> The haplo.stats package was also used to identify statistically significant associations between haplotypes and disease risks by means of generalized linear models.

## RESULTS

Demographic and clinical characteristics of the study populations are described in Table I. No deviation from the expected genotype proportion predicted by the Hardy-Weinberg equilibrium was observed for any of the polymorphisms analyzed.

The difference in genotype distribution for *ACE* I/D and -240A>T polymorphisms between PAD patients and controls was significant. A significantly higher prevalence for *ACE* D, but not -240T allele, in PAD patients was found compared with the healthy volunteers. No significant differences in genotype distribution and allele frequency were observed for the other polymorphisms investigated (Table II).

The *ACE* D and -240T alleles both significantly influenced the predisposition to PAD, even if, after Bonferroni correction for multiple testing, only the *ACE* D allele remained significantly associated with PAD (Table III). The *ACE* D allele influenced PAD predisposition with a dose-dependent effect, with odds ratios (ORs) of 1.77 for

**Table II.** Genotype distribution and allele frequencies of *ACE*, *AGT*, and *AGTR1* gene polymorphisms of the study populations

Genotype	Allele	Patients (n = 281) No. (%)	Controls (n = 485) No. (%)	P
<i>ACE</i>				
DD		94 (33.5)	127 (26.2)	...
ID		146 (52.0)	239 (49.3)	...
II		41 (14.6)	119 (24.5)	.003
	D	0.59	0.51	.001
-240TT		46 (16.4)	80 (16.5)	...
-240AT		146 (52.0)	211 (43.5)	...
-240AA		89 (31.7)	194 (40.0)	.048
	-240T	0.42	0.38	.1
<i>AGT</i>				
235TT		55 (19.6)	78 (16.1)	...
235MT		133 (47.3)	240 (49.5)	...
235MM		93 (33.1)	167 (34.4)	.3
	235T	0.43	0.41	.5
<i>AGTR1</i>				
1166CC		19 (6.8)	31 (6.4)	...
1166AC		104 (37.0)	210 (43.3)	...
1166AA		158 (56.2)	244 (50.3)	.2
	1166C	0.25	0.28	.2

*ACE*, Angiotensin converting enzyme; *AGT*, angiotensinogen; *AGTR1*, angiotensin II receptor type 1.

**Table III.** Univariate and multivariate analysis for *ACE*, *AGT* and *AGTR1* gene polymorphisms and susceptibility to peripheral arterial disease

Analysis	OR (95% CI)	P <sup>a</sup>
Univariate analysis		
Age	1.02 (0.98-1.02)	.06
Sex	0.80 (0.56-1.13)	.2
Hypertension	6.08 (4.38-8.45)	<.0001
Smoking habit	8.06 (5.71-11.32)	<.0001
Diabetes	9.15 (3.57-23.44)	<.0001
Dyslipidemia	2.79 (1.98-3.93)	<.0001
<i>ACE</i> D	1.90 (1.29-2.81)	.001 <sup>b</sup>
<i>ACE</i> -240T	1.44 (1.05-1.96)	.02 <sup>b</sup>
<i>AGT</i> 235T	1.06 (0.78-1.45)	.7
<i>AGTR1</i> 1166C	0.79 (0.59-1.06)	.1
Multivariate analysis <sup>c</sup>		
<i>ACE</i> D	2.13 (1.09-4.19)	.03

*ACE*, Angiotensin converting enzyme; *AGT*, angiotensinogen; *AGTR1*, angiotensin II receptor type 1; CI, Confidence interval; OR, odds ratio.

<sup>a</sup>Values of  $P < .05$  are significant.

<sup>b</sup>Bonferroni correction for multiple testing assessed by multiplying the nominal  $P$  value of each test by 4 (ie, the number of genetic tests conducted): *ACE* D,  $P = .004$ ; *ACE* -240T,  $P = .08$ .

<sup>c</sup>Adjusted for age, sex, and traditional cardiovascular risk factors.

ID vs II (95% confidence interval (CI), 1.18-2.67;  $P = .006$ ) and 2.15 for DD vs II (95% CI, 1.38-3.45;  $P = .001$ ), whereas no dose-dependent effect of the *ACE* -240T allele was observed. After adjustment for age, sex, and traditional risk factors, only the *ACE* D allele significantly and independently affected the susceptibility to PAD (Table III). The haplotype reconstruction analysis for the

**Table IV.** Haplotype reconstruction analysis for *ACE* gene and analysis of association with peripheral arterial disease by using the generalized linear model

Gene	Haplotype <sup>a</sup>	Frequency		Coefficient	SE	P
		PAD	Controls			
<i>ACE</i>	I-A	0.383	0.381	...	...	...
	D-A	0.193	0.237	-0.026	0.035	.5
	D-T	0.401	0.272	0.082	0.029	.005
	I-T	0.023	0.111	-0.160	0.046	.0005
Generalized linear model adjusted for cardiovascular traditional risk factors						
<i>ACE</i>	I-A	0.383	0.381	...	...	...
	D-A	0.193	0.237	0.0004	0.035	.9
	D-T	0.401	0.272	0.066	0.029	.02
	I-T	0.023	0.111	-0.127	0.052	.01

*ACE*, Angiotensin converting enzyme; *PAD*, peripheral arterial disease.

<sup>a</sup>*ACE* gene polymorphisms: I/D and -240A>T; I-A = reference haplotype.

*ACE* gene showed that the D/-240T, but not D/-240A haplotype significantly and independently influenced the predisposition to PAD (Table IV).

No significant association between *AGT* M235T and *AGTR1* 1166A>C polymorphisms and PAD was found (Table III).

We investigated the role of the *ACE* I/D polymorphism according to hypertension. The *ACE* D allele prevalence was the same in both hypertensive and normotensive patients (D = 0.59), whereas the *ACE* D allele prevalence was higher, even if not significantly, in hypertensive than in normotensive controls (D = 0.53 vs 0.49; *P* = .3; Table V).

No difference was observed in genotype distribution for all the polymorphisms analyzed, according to Rutherford categories (Table VI).

When we examined 190 of 281 PAD patients (67.6%) with no additional atherosclerotic localizations (isolated PAD), we observed a significant association between PAD and the *ACE* D (OR, 1.88; 95% CI, 1.20-2.95; *P* = .006) and -240T alleles (OR, 1.56; 95% CI, 1.09-2.23; *P* = .02). The *ACE* D allele remained significantly associated with isolated PAD after Bonferroni correction for multiple testing (*P* = .02) and after adjustment for age, sex, and cardiovascular risk factors (OR, 2.29; 95% CI, 1.14-4.61; *P* = .02). The haplotype reconstruction analysis for *ACE* gene showed that the D/-240T haplotype significantly influenced the predisposition to PAD (coefficient, 0.08 [standard error, 0.03]; *P* = .0003).

No significant association between *AGT* M235T and *AGTR1* 1166A>C polymorphisms and isolated PAD was found (*P* > .05).

## DISCUSSION

Only a limited number of studies have investigated the possible role of genetic components in the predisposition to PAD, and all the available studies have assessed one or just few genetic polymorphisms in candidate genes. The present study has evaluated the influence of four polymor-

phisms in candidate genes (*ACE*, *AGT*, *AGTR1*) as predisposing factors to PAD and has provided evidence that the *ACE* D allele represents the main modulator of the susceptibility to PAD, also in the absence of other atherosclerotic comorbidities, and that the *ACE* -240T allele has an ancillary role in predisposing to the disease. Indeed, the role of the *ACE* -240T allele is evidenced only when *ACE* D allele is present, such as in patients carrying the *ACE* D/-240T haplotype.

An association of the *ACE* D/-240T haplotype with the predisposition to the disease has also been demonstrated in PAD patients without other atherosclerotic comorbidities. No association between the polymorphisms investigated in *AGT* and *AGTR1* genes and PAD was observed.

Evidence from experimental and clinical studies shows that the RAS, through its active peptide angiotensin II, may contribute to the development and progression of atherosclerosis. The data document an association between candidate genes in RAS and CAD, AAA, and CVD,<sup>5,6,26</sup> whereas few data are available concerning PAD.<sup>2,7,8,27</sup> Findings from the present study support that the *ACE* gene and particularly the *ACE* I/D polymorphism modulates the predisposition to PAD independently of traditional risk factors.

Moreover, our results provide evidence that this polymorphism influences the susceptibility to the disease also in the absence of other atherosclerotic localizations. The relevance of this last finding might be explained by increased angiotensin II levels, which are primarily modulated by the *ACE* I/D polymorphism and in turn affect the atherosclerotic process independently of the clinical expression of the disease.

Among polymorphisms in RAS candidate genes, *ACE* I/D and -240A>T polymorphisms are of interest due to their functional roles, and the mechanisms underlying the apparent association between the *ACE* gene and atherosclerosis are intriguing. The *ACE* I/D polymorphism is known to be responsible for 47% of the total phenotypic variance of serum ACE,<sup>11</sup> even though data derived from an in vitro assay suggested that the *ACE* intron 16 sequence by itself had no effect in regulating transcription.<sup>28</sup> This result might suggest that ACE level variability is referred to another locus in linkage disequilibrium with the I/D polymorphism. Actually, experimental data from *ACE* gene expression analysis evidenced that the *ACE* D allele was associated with higher *ACE* mRNA levels.<sup>12</sup>

The other polymorphism in the promoter region of the *ACE* gene, the *ACE* -240A>T SNP, previously investigated in cardiovascular disease, has been demonstrated to account for 14% of the variability in ACE levels.<sup>16</sup>

The current case-control study is in keeping with Basar et al<sup>7</sup> but is at variance with data from Renner et al<sup>8</sup> on the *ACE* I/D polymorphism. Even if *ACE* I/D genotype distribution and allele frequency in the healthy individuals from the present study were similar to those observed by Renner et al,<sup>8</sup> the conflicting results may be due to the different sample size and clinical characteristics of patients,



**Table V.** ACE I/D polymorphism genotype distribution and allele frequency in patients and controls, according to hypertension

ACE ID polymorphism	Hypertensive, No. (%)		Normotensive, No. (%)	
	PAD, group A (n = 167)	Controls, group B (n = 99)	PAD, group C (n = 114)	Controls, group D (n = 386)
Genotype distribution				
DD	56 (33.5)	32 (32.3)	38 (33.3)	94 (24.3)
ID	86 (51.5)	40 (40.4)	58 (50.9)	188 (48.7)
II	25 (15.0)	27 (27.3)	18 (15.8)	104 (26.9)
ACE D allele frequency	0.59 <sup>a</sup>	0.53	0.59 <sup>b</sup>	0.49

ACE, Angiotensin converting enzyme; PAD, peripheral arterial disease.

<sup>a</sup>P = 0.1 for ACE D allele frequency: group A vs group B.

<sup>b</sup>P = .007 for ACE D allele frequency: group C vs group D.

**Table VI.** Genotype distribution of ACE, AGT and AGTR1 gene polymorphisms according to Rutherford categories

Genotype	Rutherford category, No. (%)				P
	2	3	4	5-6	
ACE gene					
DD	11 (55.0)	59 (32.8)	13 (32.5)	2 (22.2)	...
ID	5 (25.0)	97 (53.9)	20 (50.0)	7 (77.8)	...
II	4 (20.0)	24 (13.3)	7 (17.5)	...	.1
-240TT	7 (35.0)	24 (13.3)	7 (17.5)	3 (33.3)	...
-240AT	7 (35.0)	105 (58.3)	19 (47.5)	3 (33.3)	...
-240AA	6 (30.0)	51 (31.7)	14 (35.0)	3 (33.3)	.1
AGT gene					
235TT	5 (25.0)	30 (16.7)	11 (27.5)	5 (55.6)	...
235MT	8 (40.0)	93 (51.7)	16 (40.0)	2 (22.2)	...
235MM	7 (35.0)	57 (31.7)	13 (32.5)	2 (22.2)	.4
AGTR1 gene					
1166CC	2 (10.0)	10 (5.6)	6 (15.0)	...	...
1166AC	7 (35.0)	73 (40.6)	14 (35.0)	3 (33.3)	...
1166AA	11 (55.0)	97 (53.9)	20 (50.0)	6 (66.7)	.4

ACE, Angiotensin converting enzyme; AGT, angiotensinogen; AGTR1, angiotensin II receptor type 1.

including age, different percentage of men, smoking habits, and hypertension.

To search for the influence of other RAS candidate genes, apart from the ACE gene, on the atherosclerotic process leading to PAD, we have investigated in this study, for the first time to our knowledge, the role of the AGTR1 and AGT genes and showed no relationship with the disease. Data from our previous studies demonstrated a role for the AGTR1 1166A>C polymorphism in influencing CAD,<sup>14</sup> but not AAA disease.<sup>15</sup> Positive and negative results have been reported regarding AGTR1 gene polymorphisms in cardiovascular disease or various intermediate phenotypes, such as hypertension and intima media thickness.<sup>17,29</sup> Moreover, a recent meta-analysis of the AGT M235T polymorphism and cardiovascular risk suggests an overall weak association.<sup>30</sup> The role of the AGT gene has been also investigated in CVD and in PAD with conflicting results.<sup>2,31</sup> Therefore, data from association studies indicate an effect of the ACE gene in all localizations of

atherosclerotic process, whereas AGT and AGTR1 genes have been found to be related to coronary localization but not in extracoronary districts.

The second end point of this study was the evaluation of the influence of the polymorphisms investigated on the severity of the disease according to Rutherford categories. Our findings did not demonstrate a relationship between all the polymorphisms investigated and Rutherford categories, in keeping with results from Renner et al.<sup>8</sup> To the best of our knowledge, no data are available on the role of all the other polymorphisms analyzed in this study in modulating PAD severity.

Our study has several limitations. The main limitation is related to the fact that we were not able to perform Doppler examination with ankle-brachial index measurement as well as diagnostic procedures to evaluate asymptomatic atherosclerotic lesions in controls. A percentage of individuals with PAD are clinically asymptomatic, thus the possibility that our control group participants were disease-free cannot be excluded. Moreover, we did not provide information of the ACE phenotype. Some polymorphisms such as ACE I/D and -240A>T have functional effects on the gene product,<sup>11,12,16,28</sup> thus influencing angiotensin II levels and in turn modulating the atherosclerotic process.

## CONCLUSIONS

The present study, which shows a role for ACED/-240T haplotype in predisposing to PAD, apart from traditional cardiovascular risk factors and other atherosclerotic localizations, may contribute to identify susceptibility markers in candidate genes able to better define the pathophysiologic mechanisms leading to the atherosclerotic process.

The increasing information on genetic factors involved in atherosclerosis and in its clinical expression might contribute to the development of further treatment strategies and pharmacogenetics studies. Indeed, a beneficial effect of ACE inhibitors therapy in improving walking time in PAD patients has been demonstrated,<sup>32</sup> and it has been suggested that ACE inhibition has the potential to reduce the incidence of cardiovascular events in PAD.<sup>33</sup> Moreover, pharmacogenetics studies demonstrated a different response to ACE inhibitors related to ACE I/D polymor-

phism,<sup>34</sup> even if data from a recent meta-analysis reported conflicting results.<sup>35</sup> Therefore, results from long-term and properly designed prospective studies might contribute to perform a pharmacogenetically tailored therapy.

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## AUTHOR CONTRIBUTIONS

Conception and design: CF, ES, RA

Analysis and interpretation: CF, ES, FS, GP

Data collection: AS, GP, RP, CP

Writing the article: CF, ES, FS

Critical revision of the article: CF, CP, RA

Final approval of the article: CF, ES, FS, AS, GP, RP, CP, RA

Statistical analysis: ES, FS,

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Overall responsibility: RA

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